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GRANT NUMBER: DAMD17-94-J-4075

TITLE: Cell Signalling by a Novel SH2 Domain Protein that is Overexpressed with Her2 in Breast Cancer

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REPORT DATE: January 1996

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 1996	Annual (1 Dec 94 - 30 Nov 95)

4. TITLE AND SUBTITLE
Cell Signalling by a Novel SH2 Domain Protein that is Overexpressed with Her2 in Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4075

6. AUTHOR(S)
Benjamin L. Margolis, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
New York University Medical Center
New York, New York 10016

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

We have been studying the role of the SH2 domain protein, Grb7, in breast cancer. Studies completed this year confirmed that Grb7 is overexpressed in almost all tumors that overexpress HER2. We have generated MCF-7 breast cancer cell lines where Grb7 is overexpressed with HER2 and the EGF-Receptor/HER2 chimera. These studies confirm that Grb7 binds strongly to HER2 but overexpression of Grb7 appears to have no effect on the growth of MCF-7 cell lines or their migration in collagen gels. To clarify the role of Grb7, we are now repeating these studies in the non-transformed MCF-10A cell line that may be more sensitive to the effects of Grb7 overexpression. Other studies have examined proteins that bind to the central region of Grb7 and may be involved in signal transduction. We have identified a protein that binds to this region of Grb7 by screening bacterial expression libraries. However, the physiologic relevance of this protein/protein interaction is unclear and futher studies are being performed using bacterially expressed fusion proteins of Grb7 and radioactively labelled cell lysates. We are also generating monoclonal antibodies to Grb7 to better understand Grb7 expression and protein interactions in breast cancer and normal cells.

14. SUBJECT TERMS
Breast Cancer; HER2; Grb7; Growth Factor Receptor; Tyrosine Phosphorylation

15. NUMBER OF PAGES
15

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Ben Mayali

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INTRODUCTION

The receptor tyrosine kinase HER2 is felt to play an important role in breast cancer. It is a member of the Epidermal Growth Factor Receptor (EGFR) family of receptor tyrosine kinases that includes EGFR, HER3 and HER4. HER2 is overexpressed in approximately 20% of breast tumors and portends a poorer prognosis in patients with lymph node metastasis (1). In comedo type, ductal carcinoma in situ, HER2 overexpression occurs in greater than 80% of tumors indicating it is likely to be an initiating event (2). The rat counterpart of HER2 is known as Neu. Neu was originally isolated as an oncogene from a rat neuroectodermal tumors (3). The Neu oncogene is activated by a mutation in the transmembrane domain but these mutations have not been detected in HER2 overexpressed in breast cancer (4). The ligand for HER2 is unknown but HER2 can heterodimerize with EGFR, HER3 and HER4 and thus be activated by a family of ligands for these receptors including EGF, Heregulin and Amphiregulin (4). The signal transduction pathway of HER2 that leads to cell growth is felt to be similar to that seen with other receptor tyrosine kinases. Once activated, the receptor phosphorylates itself as well as substrate proteins on tyrosine residues. Autophosphorylation of the receptors allows the receptor to bind a variety of SH2 domain proteins involved in signal transduction.

The focus of this project is to study one of the proteins that binds to activated HER2, Grb7. Grb7 is one of the Grb family of SH2 domain proteins that was isolated by screening bacterial expression libraries with the autophosphorylated EGFR (5). It maps close to HER2 on human chromosome 17 and our group determined that it was coamplified and overexpressed with HER2 in breast cancer cell lines and a small subset of primary breast cancers (6). Grb7 is closely related to another SH2 domain protein that we have isolated and cloned called Grb10. These proteins both have SH2 domains and a conserved central domain of 300 amino acids that we have termed the GM domain because it is found in Grbs and the *C. elegans* gene, Mig-10 (7,8) The Mig-10 gene is required for embryonic migration of a sub population of neuronal cells. However unlike Grb7 and Grb10, Mig-10 does not have an SH2 domain. The central region of the GM domain contains a pleckstrin homology (PH) domain (9). The PH domain has been hypothesized to play a role in lipid or protein/protein interactions but its true role is just being elucidated. The GM domain also contains a very conserved 100 amino acid region just amino-terminal to the PH domain. Thus Grb7 and Grb10 are primarily composed of an SH2 domain and a GM domain. We know that the SH2 domain allows Grb7 to interact with HER2 and other tyrosine phosphorylated proteins. We have hypothesized that the GM domain is also involved in protein/protein interaction and that this interaction is crucial for Grb7 and Grb10 signaling. The goal of our research is determine the role of Grb7 in HER2 signal transduction in normal and pathologic states. To tackle this problem we have begun to overexpress Grb7 in breast cancer cell lines examining alterations in the behavior of the cells. We have also begun studies to delineate potential binding partners for the GM domain of Grb7 and Grb10.

BODY

Studies performed this year can be divided into three major areas. The first group of studies was the completion of a collaboration with the group at University of Texas Health Science Center at San Antonio. This involved a larger study of Grb7 expression in human breast tumors. The second group of studies that were undertaken correspond to specific aim #1 in the Statement of Work. In these studies, Grb7 was overexpressed in breast cancer cell lines in conjunction with HER2 and the effects on cell migration and the transformed phenotype were examined. The third group of studies, corresponding to specific aim #2 and #3 in the Statement of Work, were aimed at identifying proteins that interact with Grb7 and Grb10.

1) Grb7 expression in primary breast tumors. Our initial study examined approximately 70 patients with node positive tumors for Grb7 expression. These studies have now been extended to 641 node positive tumors (10). It was found that 15.1% of the patients overexpressed Grb7 in the their tumors as assessed by western blotting. In comparison, 16.4% of these tumors were positive for HER2 with a concordance of 92%. Grb7 expression was associated with a decrease in survival from 87.8 months to 58.7 months. However the effects of Grb7 and HER2 could not be dissociated because there were only a very small number of patients where HER2 was overexpressed without Grb7. Accordingly it is not clear if Grb7 alone, HER2 alone or Grb7 and HER2 are required for the worse prognosis associated with Grb7 and HER2 overexpression. These finding reinforce the importance of determining the role of Grb7 in breast cancer and signal transduction.

2) Overexpression of Grb7 in breast cancer cell lines-Specific Aim #1 in the Statement of Work: Our first step in delineating the role of Grb7 in breast cancer was to overexpress Grb7 in the breast tumors cell line, MCF-7, and look for alterations in cellular behavior. This cell line expresses little or no endogenous Grb7. We transfected MCF-7 cells with a sense and antisense Grb7 construct using the PMJ-30 mammalian expression vector. The transfection was performed using aminoglycoside aminotransferase (neo) as a selectable marker. After selection in 400 μg/ml geneticin, cell lines were cloned and Grb7 expression determined by immunoblotting. Of the initial seven clones isolated, three had significant levels of expression (Fig. 1A). The level of expression approached that seen in the breast cancer cell line, SKBR3 (Fig. 1B). Once we had obtained these cells, our next goal was to co-express the EGFR/HER2 chimera with Grb7 in the MCF-7 cells. This chimera can be activated by EGF and allows us to activate the HER2 kinase. To accomplish this task we took the MCF-7 Grb7 F4 cell line (Figs. 1A and 1B) and transfected in the EGFR/HER2 construct using hygromycin B phosphotransferase as a selectable marker. We selected for cell lines that were both geneticin and hygromycin resistant. Unfortunately we were only able to obtain one cell line that expressed both Grb7 and EGFR/HER2 despite screening several clones. In this cell line we were able to confirm our previous results that the EGFR/HER2 chimera was present in Grb7 immunoprecipitates In parallel we also produced cells that expressed only EGFR/HER2 chimera without Grb7.

At this point we tested the four different types of MCF-7 cell lines we had generated; MCF-7 parental; MCF-7 with Grb7, MCF-7 with EGFR/HER2 and MCF-7 with EGFR/HER2 and Grb7. Fig. 2 shows the phosphotyrosine content of these different cell lines after activation with EGF. It can be seen that those cells that overexpress EGFR/HER2 have increased tyrosine phosphorylation of Shc but Map kinase tyrosine phosphorylation is increased in all cell lines treated with EGF. The expression of Grb7 has no apparent effect on the pattern of phosphorylation and Grb7 itself does not appear to be tyrosine phosphorylated. We assayed the growth of these cells in soft agar in the absence and presence of EGF. We found that cells expressing the EGFR/HER2 construct formed larger colonies in soft agar but there was no effect of Grb7 on soft agar growth with or without EGFR/HER2. Another assay we performed was migration of cells in collagen gels. Again we did see an effect of EGFR/HER2 expression to increase migration in response to EGF but no effect was seen with Grb7 either alone or cotransfected with EGFR/HER2.

In summary we have completed the generation of MCF-7 cell lines as described in specific aim #1 including those overexpressing Grb7 and those overexpressing EGFR/HER2 and Grb7. One problem with these studies was that we were only able to generate one cell line that expressed both EGFR/HER2 and Grb7. Another problem is that tyrosine kinase activity is stimulated in these cells with EGF even in the absence of EGFR/HER2 expression. This indicates significant endogenous EGFR activity. Nonetheless, the studies showed that in MCF-7 cells, Grb7 overexpression does not appear to alter total phosphotyrosine content, growth or transformation nor does it appear to alter the ability of the cells to migrate in collagen gels. It seems possible that because MCF-7 cells are already transformed, it is difficult to detect an effect of Grb7. Accordingly, as outlined in the original proposal (Statement of Work, specific aim #1), we now are repeating these studies in MCF-10A cells. These cells are not transformed and require the presence of growth factors such as EGF or IGF-1 to grow. We will determine if overexpression of Grb7 in these non transformed cells will alter the growth or growth factor requirements of these cells. Studies that were not completed this year involved examining MCF-7 cells with HER2 and Grb7 in nude mice. These cell lines are in the process of being evaluated in Dr. Osborne's group at the University of Texas in San Antonio. Like the overexpression of EGFR/HER2 with Grb7, it has been difficult to generate MCF-7 cell lines with both Grb7 and HER2. We hope to overcome these problems and proceed with these studies in the second year.

3) Characterization of Grb7 binding partners - Statement of Work, Specific Aims #2 and #3 As outlined in the statement of work, specific aim #2, studies were planned in the first year to identify the specific residues on HER2 that bound Grb7. These studies were begun but have not been completed. Work from other studies have revealed the binding sites for Grb7 on two tyrosine phosphorylated proteins other than HER2 (11,12). One of these studies examined the binding to SH-PTP2, a tyrosine phosphatase while the other examined binding to Platelet Derived Growth Factor Receptor (in collaboration with our group). These studies both identify the sequence phosphotyrosine-X-asparagine as the binding site for

Grb7. This agreed with our initial speculations on the binding site on HER2 and indicates that the tyrosine at residue 1139 on HER2 is likely to be the binding site for the Grb7 SH2 domain. Our plan will be to mutate this tyrosine to phenylalanine and determine if the binding of Grb7 will be affected. However we will only proceed with this mutagenesis, once we have a cell system to monitor Grb7 activity.

As outlined in Specific Aim #3 in the Statement of Work, we have attempted to identify proteins that bind to the GM domain (central region) of Grb7 and Grb10. The Grb7 and Grb10 GM domain are 55% identical and almost surely perform a similar function. We have screened an NIH 3T3 bacterial expression library with the GM domain of Grb10 because our best expression library was from NIH 3T3 cells, where Grb10, but not Grb7, is expressed. The central domain of the protein was expressed as glutathione-s-transferase (GST) fusion protein that contains a protein kinase A phosphorylation site. This fusion protein can be radioactively labelled with protein kinase A and γ^{32}P-ATP (13). After screening 800,00 plaques with this Grb10 probe, one positive clone was identified and purified. Sequencing revealed this clone to represent a partial sequence of the protein Nopp140, a serine rich highly phosphorylated protein that may be involved in nuclear import (14). Antibodies to this protein were obtain from Dr. U.T. Meier and attempts were made to detect Nopp140 in Grb7 or Grb10 immunoprecipitates. However no interaction could be demonstrated between Grb7 or Grb10 and Nopp140 in living cells. Accordingly, the biological significance of this interaction is still open to question and may be an artifact of the cloning procedure.

It seemed possible that proteins might require modification (such as phosphorylation) in order to bind the GM domain and we might not be able to isolate interacting proteins using expression cloning. Accordingly, we have began to isolate proteins from breast cancer cells that will bind to the Grb7 GM domain. The basic approach we have used involves labelling cells with ^{35}S-methionine or ^{32}P-orthophosphate and then either immunoprecipitating the lysates with anti-Grb7 antibody or binding the lysates to GST fusion proteins containing regions of Grb7. Preliminary results from these experiments suggested the presence of a 35 kDa protein seen both in immunoprecipitation and GST pull down assays (Fig. 3). Further experiments will be necessary to confirm this result and indicate if this is a true binding partner for the GM domain. The possibility was also considered that the GM domain of Grb7 might be involved in lipid interactions. However we find more than 90% of Grb7 to be cytosolic and not bound to membranes.

CONCLUSIONS

As outlined we have completed or initiated several points of the proposed specific aims. The studies we have completed have laid the ground work for further studies to be carried out in the next year.

Specific Aim #1. We have generated MCF-7 cell lines with Grb7 and EGFR/HER2 and have performed growth and migration assays. In MCF-7 cells,

we see no effect of Grb7 on migration or soft agar growth. However it is possible that MCF-7 might not be the best cell line for these type of experiments and the transfections are now being repeated in MCF-10A cells. We think these MCF-10A cells will assist in identifying a functional readout for Grb7. Furthermore these cells have been extensively studied by Dr. Steve Ethier at The University of Michigan who has agreed to collaborate with us on these projects. He has examined the effect of HER2 overexpression on these cells previously (15).

Specific Aim #2. We plan to mutate the putative Grb7 binding site at Y1139 of HER2 to phenylalanine to determine if this will impair HER2 signaling. This specific aim will be given a lower priority until we have a better cell system to study Grb7 signaling.

Specific aim #3. We isolated a protein, Nopp140, by bacterial expression cloning that bound to the central domain of Grb10. Unfortunately this protein did not appear to bind to Grb10 in living cells forcing us to reevaluate our approach to delineating proteins that bind GM domains. We have redirected these studies to analyze protein/protein interactions in radiolabelled living cells hypothesizing that modifications of proteins, such as phosphorylation, will be necessary for the interaction. We have two plans to improve our ability to detect such interactions by coimmunoprecipitation. First, we plan to express Grb7 containing a myc epitope in MCF-10A cells. We will be able to immunoprecipitate Grb7 with a monoclonal antibody directed against the myc epitope as well as with polyclonal antibodies to Grb7 that we had been using previously. This will allow us to identify proteins that interact with Grb7 regardless of the specific antibody used. Second, we plan to generate mouse monoclonal antibodies directed against Grb7. We have begun to inject mice with a GST-Grb7 construct and will screen the mouse serum and hybridoma supernatants with a histidine tagged Grb7. The injections and fusions will be performed at the hybridoma core facility at the University of Michigan. These monoclonals may not only be useful for Grb7 immunoprecipitation but hopefully will also be useful in immunohistochemistry. It will be very important to analyze Grb7 expression by immunohistochemistry in breast cancer especially comedo style ductal carcinoma in situ where HER2 is overexpressed. In conjunction with the coimmunoprecipitation experiments we will continue to use the GST fusion protein pull down assay to determine which regions of Grb7 are responsible for interaction with proteins seen in the coimmunoprecipitations.

Specific Aim #4. Work has not yet begun on this portion of the project and awaits the development of a better cell system to study Grb7.

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APPENDIX

FIGURES and LEGENDS

Fig. 1. A. Generation of MCF-7 cells overexpressing Grb7. MCF-7 cells were transfected with sense or antisense Grb7 cDNA using aminoglycoside phosphotransferase as a resistance marker. Clones were selected that were resistant to geneticin and lysates prepared. These lysates were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-Grb7 #222 antibody. R represents clones that received GRB7 cloned in the Reverse (anti-sense) direction while F represents those that received GRB7 in the Forward (sense) direction. C represents lysate from an NIH 3T3 cell line transfected with Grb7. Although this blotting had several background bands, the overexpression of GRB7 was detected in cell lines F1, F3, F4. **B. Confirmation of GRB7 overexpression.** Due to the background with antibody 222, the lysates from cell lines R1, F1, F3, F4 were blotted with another Grb7 antibody, #188, to confirm overexpression. Also shown is an equal amount of lysate from the SKBR3 cell line where Grb7 is overexpressed. F1, F3 and F4 expressed amounts of Grb7 similar to that seen in the SKBR3 cell line.

Fig. 2. Stimulation of tyrosine phosphorylation by EGF in MCF-7 cells transfected with EGFR/HER2, Grb7 or both. MCF-7 cells, either parental or stable transfectants were stimulated with 250 ng/ml EGF for 5 minutes. Lysates were prepared, separated by SDS-PAGE, transferred to nitrocellulose and blotted with anti-phosphotyrosine antibodies. The major tyrosine phosphorylated proteins have been identified as p52 Shc and Map kinase.

Fig. 3. A. Binding of a 35 kDa ^{35}S-labelled protein to Grb7 fusion proteins and Grb7 immunoprecipitates. MCF-7 cells were labelled with 100 μCi/ml ^{35}S-Methionine for 4 hours. Cells were then stimulated with EGF and lysates prepared. The lysates were bound to fusion proteins consisting of glutathione-s-transferase (GST) alone, GST coupled to the PH domain of Grb7 and GST coupled to the GM domain of Grb7. The lysates were also immunoprecipitated with anti-Grb7 antibody (affinity purified #222) or pre-immune control. The bound proteins were separated by SDS-gel and detect by autoradiography after gel treatment with Amplify (Amersham). The arrow points to a 35 kDa protein seen in the GST-Grb7-PH and GST-Grb7-GM pull down assays as well as in Grb7 immunoprecipitates. **B. Coimmunoprecipitation of a 35 kDa protein with Grb7 in ^{32}P-labelled cells.** MCF-7 cells (parental or transfected with EGFR/HER2 and Grb7) were labelled with 1 mCi/ml of ^{32}P-orthophosphate for three hours. Immunoprecipitation was then performed with anti-Grb7 antibody (affinity purified #222). After washing six times with HNTG (Hepes, 20 mM pH 7.5, 150 mM NaCl, 10 % glycerol and 0.1% Triton X-100), the samples were separated by SDS-PAGE and subjected to autoradiography. The coimmunoprecipitating 35 kDa protein is more clearly seen here than in the ^{35}S-Methionine labeled cells.

Fig. 1A

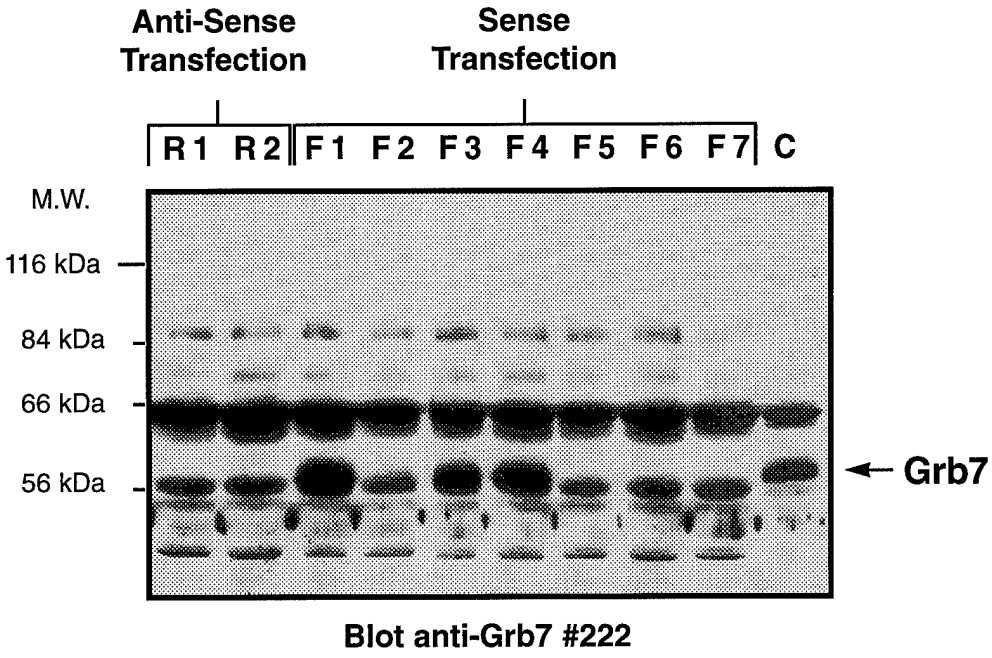

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Fig. 1B

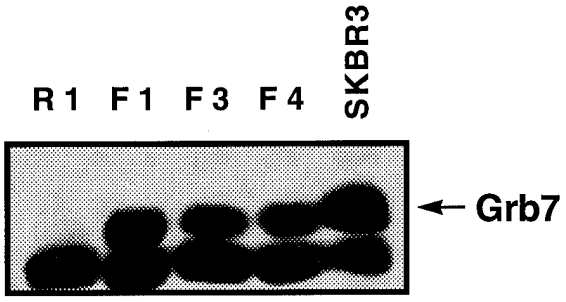

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Fig. 2

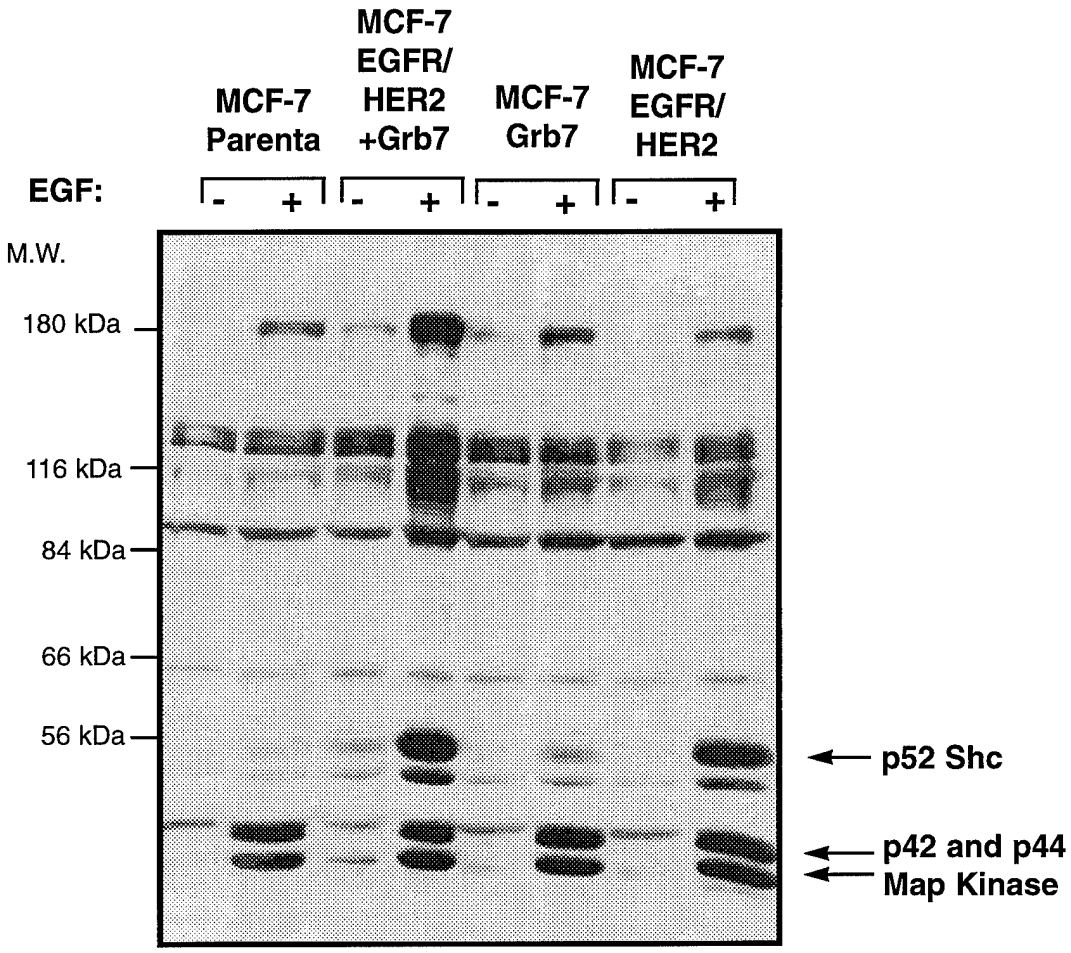

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Fig. 3A

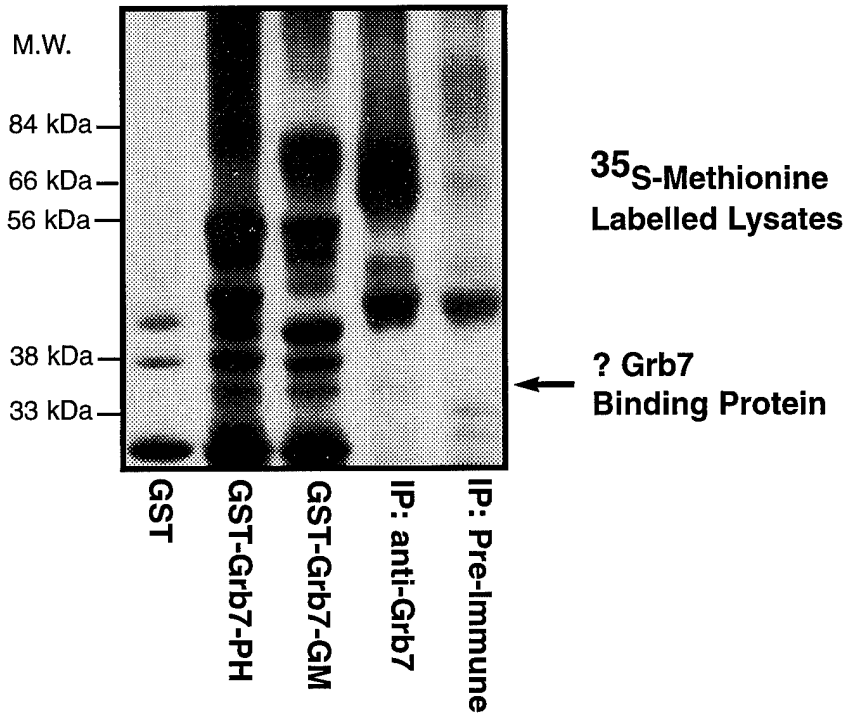
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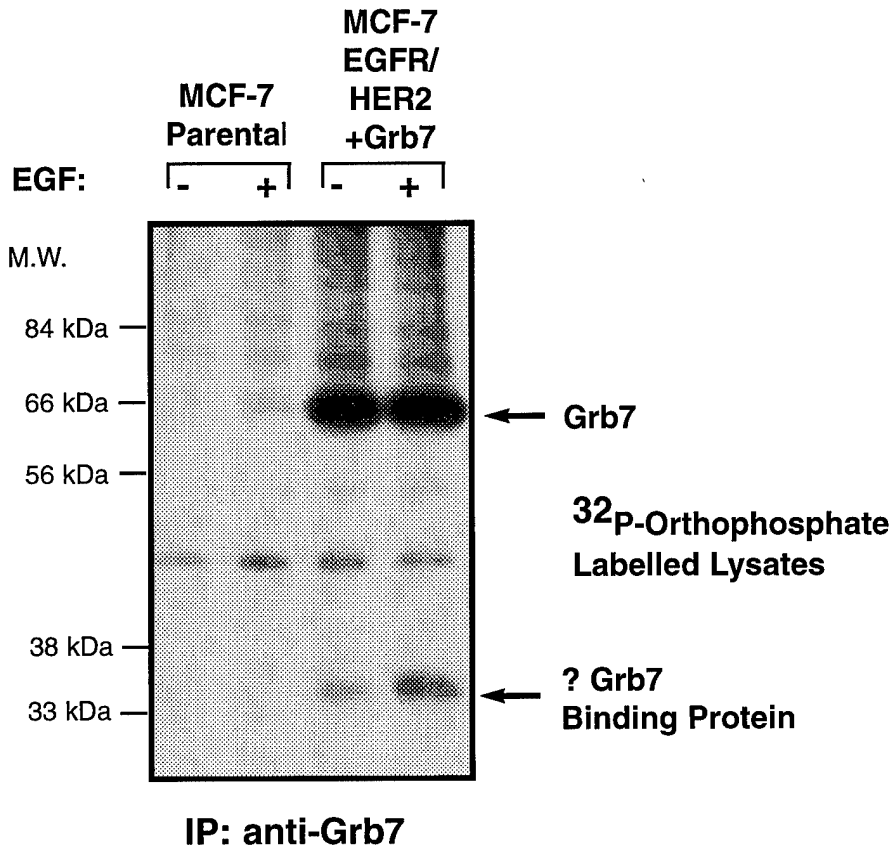
Fig. 3B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38 kDa
33 kDa
Grb7
32P-Orthophosphate
Labelled Lysates
? Grb7
Binding Protein
IP: anti-Grb7